Measurement of Suppressor Transfer RNA Activity

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8. Animals were maintained on a 12:12 hour light-dark cycle (lights on at 0730) at about 22°C. Experimentation began at 0830. Animals were tested consecutively in batches of seven scrambled with respect to solution drunk and genetic background so that at least one representative of each group was included in each batch.
9. Animals were placed for 1 minute into a 20 by 20 by 40 cm plexiglass chamber whose floor was a copper hot plate covered by cardboard insulation. After this acclimation period the insulation was withdrawn and time was measured by stopwatch until the animal licked one of its hind paws or 45 seconds elapsed. The animal was then returned to its cage. Two observers were used to determine occurrence of the pain response. The hot plate was maintained at 53°C by a Fried Electric heater and thermostat, which circulated heated water through uniformly distributed tubing beneath the copper floor. Temperature was also monitored independently of the thermostat by a thermometer, the tip of which was submerged in the heated water.
10. Mean daily consumption of water was 21 and 25 ml and mean initial body weight was 263 and 255 g for LC2-Lo and LC2-Hi rats, respectively. No significant differences in gains in body weight between the two lines and among the different drinking groups were detected during the study. The mean amount of food consumed in 72 hours was 50 and 52 g for LC2-Lo and LC2-Hi rats, respectively. No significant differences in the quantity of food consumed in a second food consumption measurement were detected between lines or drinking groups.
11. The opioid form of stress-induced analgesia is partly defined by its sensitivity to blockade by naloxone [J. W. Lewis, J. T. Cannon, J. C. Liebeskind, *Science* **208**, 623 (1981)].
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Abstract. *Transfer RNA (tRNA) suppression of nonsense mutations in prokaryotic systems has been widely used to study the structure and function of different prokaryotic genes. Through genetic engineering techniques, it is now possible to introduce suppressor (Su^+) tRNA molecules into mammalian cells. A quantitative assay of the suppressor tRNA activity in these mammalian cells is described; it is based on the amount of tRNA-mediated readthrough of a terminating codon in the influenza virus NS1 gene after the cells are infected with virus. Suppressor activity in L cells continuously expressing Su^+ ($tRNA^{tyr}$) was 3.5 percent and that in CV-1 cells infected with an $SV40^-$ Su^+ ($tRNA^{tyr}$) recombinant was 22.5 percent.*

The availability of SV40 vectors containing amber suppressor (Su^+) $tRNA^{tyr}$ (tyrosine transfer RNA) genes (*1*) and the establishment of eukaryotic cell lines (*1*) expressing functional suppressor tRNA's give promise of a new approach to the genetic analysis of cellular and viral genomes. Development of this new technology requires a rapid and quantitative assay for the level of suppressor activity in a particular system. The genetic structure of influenza virus provides an ideal system to measure the suppressor tRNA mediated readthrough of stop (terminating) codons.

Extensive studies on the structure of the NS gene of different influenza virus field isolates have shown that the NS1 gene products may vary in length. For example, the NS1 protein of the 1947 strain A/FM/1/47 is only 202 amino acids while the A/USSR/90/77 virus NS1 gene product contains 237 amino acids (*2*). Another field virus isolate, A/PR/8/34 virus, has a long NS1 protein (230 amino acids) (*2*). The NS genes of these three viruses all terminate the NS1 protein via a UGA (U, uracil; G, guanine; A, adenine) codon (opal codon). In contrast, A/CAM/46, another influenza virus field isolate, directs the synthesis of a 216 amino acid NS1 protein terminating with an amber codon (UAG). This amber stop signal is followed downstream by a UGA triplet at amino acid position 237 (*2*). Therefore, amber suppression is expected to produce an NS1 polypeptide with an additional 20 amino acids. Since NS1 polypeptides of length 237 are found in other field variants, it is likely that both the terminated (at position 216) and suppressed (at position 237) proteins would be stable.

Virus stocks of SV40 recombinants containing active amber Su^+ $tRNA^{tyr}$ genes have been described (*1*) and have been shown to suppress a nonsense (UAG) mutation in the thymidine kinase gene of herpes simplex virus mutants (*3*).

Table 1. Suppression of termination at NS1 amber codon. The SV40 recombinants and L cell lines used have been described (*1*). The amber readthrough was measured by infection of cells with influenza A/CAM/46 virus as described in the text. The values of the influenza virus NS1 readthrough protein represent the average of two independent experiments. Conditions for infection of CV-1 cells and for L cells were as described for Fig. 1, lanes 7 and 8.

Cell	Amount (%)
CV-1 plus Su^+ SV40 recombinant	22.5
CV-1 plus Su^- SV40 recombinant	< 0.5
L cell control	< 0.5
L cell line 37	3.6
L cell line 39	3.4

Infection of CV-1 cells with these stocks results in accumulation of the Su^+ tRNA to 2 to 5 percent of the total cellular tRNA.

To test the readthrough of the amber codon in the NS1 coding region, CV-1 cells were infected with A/CAM/46 virus in the presence of SV40 recombinants containing a Su^+ or a Su^- tRNA gene. Infection of CV-1 cells for 24 hours with the SV40 recombinants containing the Su^+ tRNA gene resulted in the production of suppressor activity to give partial readthrough of the NS1 protein (Fig. 1, lane 8, arrow). In contrast, infection with the equivalent Su^- SV40 recombinant did not permit synthesis of such a polypeptide in A/CAM/46 virus infected CV-1 cells (Fig. 1, lane 7). Similar results were obtained when CV-1 cells were infected with the SV40 recombinants for 48 hours prior to the addition of A/CAM/46 virus (Fig. 1, lanes 9 and 10). However, no suppressor tRNA activity was detectable in cells that were coinfected at the same time with the SV40 recombinants and A/CAM/46 virus (Fig. 1, lane 6). The gel migration of the additional protein band in lanes 8 and 10 of Fig. 1 is in agreement with the predicted length of the readthrough product of the NS1 gene of A/CAM/46 virus.

The identity of this readthrough product, designated by the arrow in Fig. 1, was confirmed in a subsequent experiment with a monospecific rabbit antiserum to bacterially synthesized NS1 protein (*4*). This antiserum precipitates both the NS1 polypeptide as well as the suppressed readthrough product from an infected cell extract (Fig. 2, lane 3). In contrast, when cells are coinfected with the Su^- SV40 recombinants and A/CAM/46 virus, only the nonsuppressed NS1 polypeptide is present (Fig. 2, lanes 4 and 6). Gel separation of the labeled NS1 polypeptide and its readthrough product and analysis of the gel slices revealed a 15 to 25 percent readthrough of the amber codon in the CV-1 cells infected with A/CAM/46 virus and the Su^+ SV40 recombinants. Optimal readthrough occurs if the influenza virus infection follows an 18- to 30-hour preliminary incubation with the Su^+ SV40 recombinants (data not shown). Use of a tenfold concentrated Su^+ SV40 recombinant virus stock did not increase the amount of the readthrough product. However, a fivefold dilution of the Su^+ SV40 recombinant preparation used to infect CV-1 cells did lead to a 50 percent reduction of the amber codon suppression.

Amber suppression in cell lines carry-

ing functional suppressor tRNA genes was also measured by infection with A/CAM/46 virus. These cell lines were established by microinjection of Su^+ $tRNA^{tyr}$ recombinant DNA into L cells containing three selectable genes each carrying an amber nonsense mutation (*1*). Cell clones established by selection for any one of the three genes were also positive for suppression of the other two nonsense mutations. Two of these L cell lines, 37 and 39, and the parental Ltk^- cell were infected with A/CAM/46 virus. Several experiments revealed a 3.4 and 3.6 percent readthrough efficiency in cell line 39 and cell line 37, respectively (Table 1). Therefore, the level of amber suppression in the established Su^+ L cells appears to be lower than that in Su^+ SV40 recombinant infected CV-1 cells.

Our experiments permit rapid quantification of the amber suppressor tRNA activity in CV-1 and L cells containing the suppressor $tRNA^{tyr}$ genes. Such a quantitative analysis of functional suppressor activity was previously not possible (*1*). Since most mammalian cells will express viral proteins after infection with influenza viruses grown in embryonated eggs, our technique can be used for a number of cell systems. The assay should also be useful in analyzing cells containing other suppressor tRNA genes that insert an amino acid other than tyrosine. The carboxyl terminal of the NS1 protein is not essential for its function (*2*), and any amino acid replacement of the amber codon will probably not affect the level of the NS1 readthrough product found in cells.

The efficiency of suppression of termination observed at a particular nonsense codon should depend on the level of charged Su^+ tRNA, the effect of codon context (*5*), and the level of release factors (*5*). Different amber nonsense codons might be recognized with different efficiencies by a suppressor tRNA. In this regard, it is reassuring that when another influenza virus strain, A/Ann Arbor/8/60, with an NS1 gene terminating in an amber codon is used, similar levels of suppression are observed (data not shown). The selection protocol used to establish the L cell lines containing integrated Su^+ $tRNA^{tyr}$ genes revealed that cells with only 3 percent of wild-type levels of thymidine kinase or guanine ribosyl transferase activity were able to survive in the appropriate selection medium (*6*). Thus the 3.5 percent level of suppression observed in these cell lines is not unexpected and should provide an adequate level of suppressor activity for growth of virus mutants with amber codons in catalytic functions.

The influenza virus system described above may also be useful in determining opal and ochre suppressor activity. The NS gene of another influenza virus isolate, A/USSR/90/77 virus (*2*), has a UGA

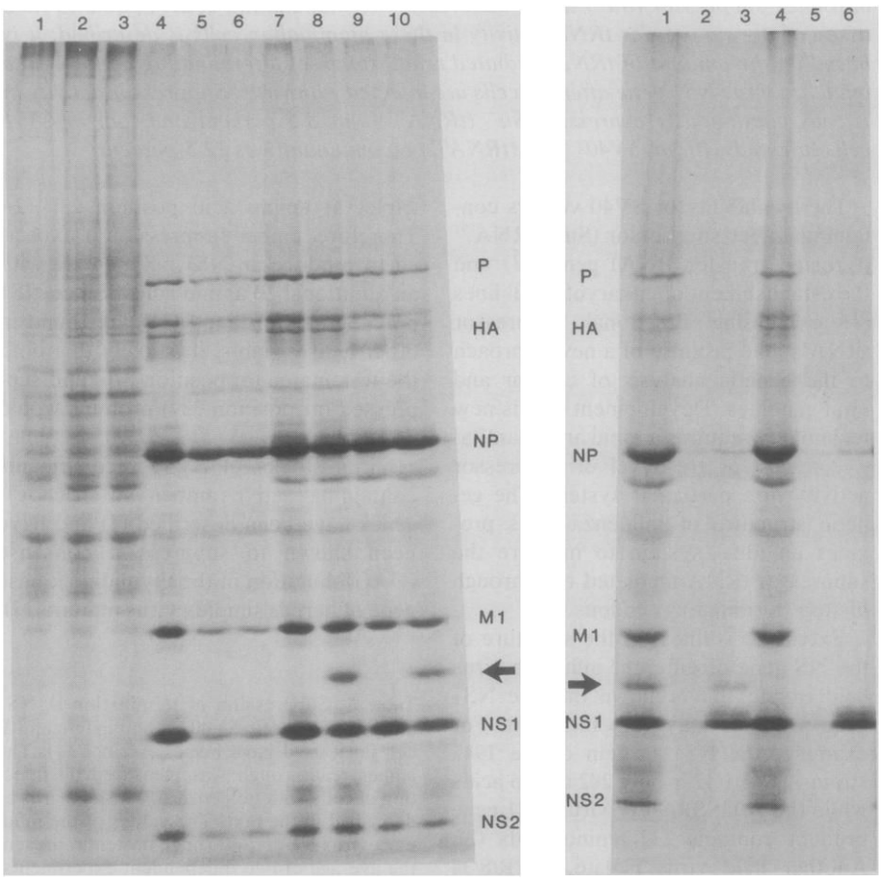

Fig. 1 (left). Suppressor tRNA activity in SV40 recombinant virus infected CV-1 cells detected by suppression of the influenza virus NS1 polypeptide. CV-1 cells (3×10^6 cells) were pulse-labeled (200 μCi of L-[^{35}S]methionine) by incubation for 1 hour at 37°C in warmed protein labeling medium consisting of Hanks balanced salt solution (Ca and Mg free) with 0.5 percent $NaHCO_3$ and 0.2 percent glucose. After being labeled, cells were washed with phosphate-buffered saline (PBS) and treated with lysis buffer containing 2 percent sodium dodecyl sulfate (SDS), 2 percent mercaptoethanol, 2 percent phenylmethylsulfonyl-fluoride (PMSF), 120 m*M* tris, 64 m*M* H3PO4 and 20 percent glycerol. Samples were boiled and subjected to electrophoresis on a gradient polyacrylamide gel (7 to 14 percent); the discontinuous buffer system of Maizel was used (*8*). At the indicated times cells (*4*) were infected with influenza A/CAM/46 virus at a multiplicity of infection (MOI) of 10. (Lane 1) Uninfected cells. (Lane 2) Cells infected for 30 hours with Su^- SV40 recombinant SV-tT-2/SV-rINS-7 (*1*). (Lane 3) Cells infected for 30 hours with Su^+ SV40 recombinant SV-tT-2(Su^+)/SV-rINS-7 (*1*). (Lane 4) Cells infected for 6 hours with A/CAM/46 virus. (Lane 5) Cells coinfected for 6 hours with A/CAM/46 virus and Su^- SV40 recombinant SV-tT-2/SV-rINS-7. (Lane 6) Cells coinfected for 6 hours with A/CAM/46 virus and Su^+ SV40 recombinant SV-tT-2(Su^+)/SV-rINS-7 (*1*). (Lane 7) Cells were first infected with Su^- SV4 recombinant SV-tT-2/SV-rINS-7 and then superinfected 24 hours later with A/CAM/46 virus and held for 6 hours. (Lane 8) Cells first infected with Su^+ SV40 recombinant SV-tT-2(Su^+)/SV-rINS-7 and then superinfected 24 hours later with A/CAM/46 virus and held for 6 hours. (Lane 9) Cells first infected for 48 hours with Su^- SV40 recombinant SV-tT-2/SV-rINS-7 and superinfected with A/CAM/46 virus for an additional 6 hours. (Lane 10) Cells preinfected for 48 hours with Su^+ SV40 recombinant SV-tT-2(Su^+)/SV-rINS-7 and superinfected with A/CAM/46 virus for 6 hours more. The molecular weights of the P, HA, NP, M1, NS1, and NS2 polypeptides are 82K to 86K, 77K, 56K, 28K, 23K, and 14K, respectively. Fig. 2 (right). Immunoprecipitation of NS1 and the NS1 readthrough product from extracts of CV-1 cells infected with SV40 recombinants and A/CAM/46 influenza virus. Twenty-four hours after CV-1 cells were infected with SV40 recombinants, A/CAM/46 virus was added and incubation continued for another 6 hours. Cells were labeled with [^{35}S]methionine, and extracts were prepared as described (*8, 9*). Proteins were immunoprecipitated by the *Staphylococcus aureus* method with a monospecific rabbit antiserum to bacterially synthesized NS1 protein (*8, 9*). (Lane 1) Cells coinfected with SV-tT-2(Su^+)/SV-rINS-7 (*1*) and A/CAM/46 virus. (Lane 2) Cell extract as in lane 1 after immunoprecipitation with normal rabbit serum. (Lane 3) Cell extract as in lane 1 after immunoprecipitation with the rabbit antiserum to NS1. (Lane 4) Cells coinfected with SV-tT-2/SV-rINS-7 and A/CAM/46 virus. (Lane 5) Cell extract as in lane 4 after immunoprecipitation with normal rabbit serum. (Lane 6) Cell extract as in lane 4 after immunoprecipitation with the rabbit antiserum to NS1.

triplet at amino acid position 237 and an amber codon at amino acid position 249; the NS gene of A/Berk/1/68 virus terminates NS1 with an ochre triplet (UAA) at position 220 followed by a UGA triplet 17 amino acids downstream (*2*). Thus, these two viruses may be conveniently used for the analysis of opal and ochre suppressor activity, respectively. Finally, the analysis system described here may not only be useful for measuring nonsense suppressor activity in cells transformed by genetic engineering techniques, but also for detecting suppressor activity in eukaryotic cells containing natural nonsense suppressor tRNA's (*7*).

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Corticotropin Releasing Factor Decreases Postburst Hyperpolarizations and Excites Hippocampal Neurons

Abstract. *Corticotropin releasing factor in concentrations of 15 to 250 nanomoles per liter increased the spontaneous discharge frequency and decreased the size of hyperpolarizations after burst discharges in CA1 and CA3 pyramidal neurons of rat hippocampal slices. Concentrations greater than 250 nanomoles per liter also depolarized the cells. These excitatory actions of corticotropin releasing factor may involve a novel calcium-dependent process.*

Endogenous hypothalamic factors capable of releasing adrenocorticotropic hormone (ACTH) and β-endorphin from the pituitary have been recognized for some time (*1*). However, only recently has a corticotropin releasing factor (CRF) been purified, sequenced, and synthesized (*2*). Synthetic CRF releases ACTH and β-endorphin from the pituitary and, when centrally administered, activates the sympathetic nervous system (*3*), suggesting that CRF may be a key hormone in mobilizing the organism under stress (*2*). This view is supported by the recent findings that centrally administered CRF causes hyperactivity and arousal in rats (*4*) and that CRF immunoreactivity is seen in cell bodies

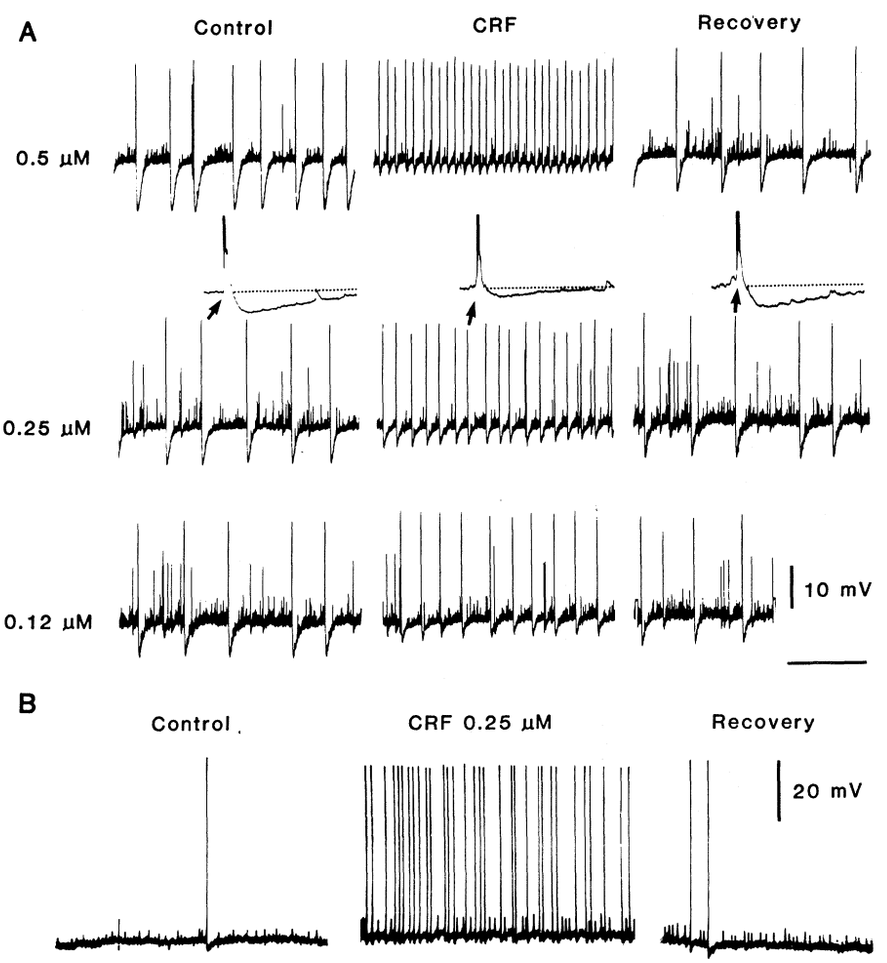

Fig. 1. Effects of CRF on spontaneous spikes and transmembrane properties of pyramidal neurons. (A) Dose-dependent increases in firing and reductions of postburst afterhyperpolarizations by CRF in a CA3 neuron. Spontaneous large spikes followed by afterhyperpolarizations are actually bursts of multiple spikes that appear as single attenuated spikes because of the slow speed and rise time of the chart recorder. Arrows indicate representative bursts displayed at faster speed (insets). Dotted lines represent the baseline potentials in each condition. The spike firing rate increases at all CRF concentrations. (B) Oscillographs from a CA1 neuron: 0.25 μ*M* CRF increases the spike discharge rate and slightly depolarizes (about 3 mV) this cell. There is an apparent increase in the rate and size of activity subthreshold for somatic spike generation (excitatory postsynaptic potentials or dendritic spikes). Horizontal calibration bars are 20 seconds in (A), 0.8 second in the insets, and 2 seconds in (B). All CRF records were obtained within 2 to 5 minutes of control records; recovery records were taken at 10 to 40 minutes after CRF washout.